Estrogen Sensitivity of Target Genes and Expression of Nuclear Receptor Co-Regulators in Rat Prostate after Pre- and Postnatal Exposure to the Ultraviolet Filter 4-Methylbenzylidene Camphor

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Background and objectives: In previous studies, we found that the ultraviolet filter 4-methylbenzylidene camphor (4-MBC) exhibits estrogenic activity, is a preferential estrogen receptor (ER)-β ligand, and interferes with development of female reproductive organs and brain of both sexes in rats. Here, we report effects on male development.

Methods: 4-MBC (0.7, 7, 24, 47 mg/kg/day) was administered in chow to the parent generation before mating, during gestation and lactation, and to offspring until adulthood. mRNA was determined in prostate lobes by real-time reverse transcription–polymerase chain reaction and protein was determined by Western blot analysis.

Results: 4-MBC delayed male puberty, decreased adult prostate weight, and slightly increased testis weight. Androgen receptor (AR), insulin-like growth factor-1 (IGF-1), ER-α, and ER-β expression in prostate were altered at mRNA and protein levels, with stronger effects in dorsolateral than ventral prostate. To assess sensitivity of target genes to estrogens, offspring were castrated on postnatal day 70, injected with 17β-estradiol (E_2; 10 or 50 μg/kg, sc) or vehicle on postnatal day 84, and sacrificed 6 hr later. Acute repression of AR and IGF-1 mRNAs by E_2, studied in ventral prostate, was reduced by 4-MBC exposure. This was accompanied by reduced co-repressor N-CoR (nuclear receptor co-repressor) protein in ventral and dorsolateral prostate, whereas steroid receptor coactivator-1 (SRC-1) protein levels were unaffected.

Conclusions: Our data indicate that 4-MBC affects development of male reproductive functions and organs, with a lowest observed adverse effect level of 0.7 mg/kg. Nuclear receptor coregulators were revealed as targets for endocrine disruptors, as shown for N-CoR in prostate and SRC-1 in uterus. This may have widespread effects on gene regulation.

Key words: androgen receptor, development, estrogen receptors, gene expression, insulin-like growth factor-1, 4-methylbenzylidene camphor (4-MBC), N-CoR, prostate, puberty, SRC-1, UV filter. *Environ Health Perspect* 115(suppl 1):42–50 (2007). doi:10.1289/ehp.9134 available via *http://dx.doi.org/* [Online 8 June 2007]

Exposure to endocrine-active chemicals has been associated with developmental and reproductive abnormalities in wildlife and is suspected to interfere with human endocrine systems. Screening of endocrine activity has focused on industrial, pharmaceutical, and agricultural chemicals. However, recent studies indicate that endocrine-active chemicals are also found among preservatives, antioxidants and ultraviolet (UV) filters that are constituents of cosmetics. Some UV filters exhibit estrogenic or antiandrogenic activity (Holbech et al. 2002; Inui et al. 2003; Ma et al. 2003; Mueller et al. 2003; Schlumpf et al. 2001a; Schreurs et al. 2002; Tinwell et al. 2002). 4-MBC, a UV filter in sunscreens, and 3-benzylidene camphor, a structurally related UV absorber, are interesting because they exhibit preference for estrogen receptor (ER)-β (Schlumpf et al. 2004a). UV filters also interfere with the mammalian thyroid axis (Schmutzler et al. 2004).

These compounds represent a new class of endocrine-active chemicals. The lipophilic, high-production volume substances with an increasingly diverse spectrum of use as cosmetic constituents and technical UV absorbers are released into the environment and found in lakes and rivers and in fish (Balmer et al. 2005; Nagtegaal et al. 1997; Poiger et al. 2004). Their main sources are sewage treatment plant (STP) effluents (Plagellat et al. 2006). UV filters in sunscreens may reach humans and animals via the food web, direct application of cosmetics (Hayden et al. 1997; Janjua et al. 2004), and contacts with UV stabilizers in plastics, clothing, curtains, and food (Kawamura et al. 2003).

We investigated effects of pre- and postnatal exposure to 4-MBC in rats. Data on female littermates and brain of both sexes are published (Durrer et al. 2005; Maerkel et al. 2005, 2007). Here, we report on effects in male offspring. The treatment period overlaps with periods of urogenital tract development during pre- and early postnatal life that are sensitive to unbalanced hormonal status. Prostate development is testosterone dependent and sensitive to estrogens (Vom Saal et al. 1997. Woodham et al. 2003). Perinatal estrogen exposure permanently imprints prostate development and is associated with increased incidence of prostate hyperplasia, dysplasia, and adenocarcinoma. The investigation revealed effects on classic toxicologic end points, expression of estrogen-regulated genes at mRNA and protein levels, estrogen sensitivity of target genes, and nuclear receptor co-repressor levels.

Materials and Methods

Chemicals. 3-(4-methylbenzylidene) camphor (4-MBC; Eusolex 6300 CAS no. 36861-47-9, purity 99.7–99.9%) was purchased from Merck (Dietikon, Switzerland) and 17β-estradiol (E_2) from Calbiochem (Lucerne, Switzerland).

Experimental animals. Long Evans rats purchased from Møllegaard Breeding and Research Centre (Ejby, Denmark) were bred under controlled illumination (lights on 0200–1600 hours) and temperature (22°C ± 1°C) with free access to food and water. The animal facility is run by the Institute of Laboratory Animal Sciences, University of Zurich. Microbiological checks are performed every 3 months.

Animal welfare. Animals were treated humanely and with regard for alleviation of suffering, in line with the Swiss Law for the Protection of Animals (Tierschutzgesetz, 9 March 1978) and the Ethical Guidelines for Animal Experimentation of the Swiss Academy for Medical Sciences. The project (no. 132/2003) was approved and regularly supervised by the Committee for Animal Experimentation of the State of Zurich and the Veterinary Office of the State of Zurich.

Experimental design. **Food pellets containing 4-MBC.** Food pellets with 4-MBC were prepared by Provimi Kliba AG (Kaiseraugst, Switzerland). 4-MBC was dissolved in cold-pressed soy oil (Morga; Ebnat-Kappel, Switzerland) and added to Provimi Kliba chow 3340 to achieve 4-MBC concentrations of 0.01, 0.1, 0.33, and 0.66 g/kg chow, yielding an average daily intake of 0.7, 7, 24, or 47 mg/kg/day. Control pellets consisted of the

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same matrix (Provimi Kliba 3340) with 1% of soil oil added. The soy oil preparation (Morga) was devoid of detectable amounts of phytoestrogens (manufacturer's information).

4-MBC treatment. We designed the study to mimic exposure through the food chain. It followed the design of a two-generation test without F_2. Males and females of the parent generation (5–6 weeks old) were fed for at least 10 weeks before mating with chow containing 4-MBC (0.01, 0.1, 0.33, and 0.66 g/kg chow) or with control chow. Treatment continued throughout pregnancy and lactation and in the offspring until adulthood. Pregnant dams were weighed on gestational day (GD)1 (GD1 = 24 hr after onset of mating), GD7, 14, and 22. Offspring were counted on the day of birth [postnatal day (PND)1 = GD23), and sexed and adjusted to 8–10 animals/litter on PND2. Body weight was recorded on PND2, 4, 6, 9, 12, 13, 14, at puberty onset, and in adulthood. After weaning (PND28), males and females were raised separately. Additional parameters studied include sex ratio, survival rate, righting reflex, anogenital distance, and eye opening (Durrer 2004). Onset of puberty (preputial separation, vaginal opening) was investigated in males from PND41 and in females from PN30. Experiment A, control A, together with 7, 24, and 47 mg/kg 4-MBC, and experiment B, control B, together with 0.7 mg/kg 4-MBC, were run separately (Table 1).

Male offspring used for molecular biological analysis of prostate were taken from the same litters as the females (Durrer et al. 2005). Brain regions of the same male and female offspring were also analyzed (Maerkel et al. 2005, 2007). For analysis of male reproductive organ weights and puberty, offspring from additional litters were included.

Adult offspring under baseline conditions. Adult offspring under baseline conditions were raised without further experimental manipulation except 4-MBC treatment and sacrificed at 12 weeks by decapitation during light ether anesthesia. The ventral lobe of prostate (ventral prostate) and the combined dorsal + lateral lobes (dorsolateral prostate) were dissected according to J. Ashby (personal communication). Dorsal and lateral lobes were cut inside the borderline with adjacent tissue to avoid contaminations and achieve identical preparation times for ventral and dorsal + lateral lobes. Therefore, wet weight was determined only for ventral lobe. Weights of dorsal + lateral and ventral lobes were determined in 13-month-old 4-MBC–exposed offspring in an additional series (Supplemental material; http://www.ehponline.org/members/2007/9134/suppl.pdf). Both tissue pieces were immediately frozen and stored in liquid nitrogen.

Estrogen challenge experiment. Acute responsiveness of estrogen target genes was analyzed in castrated rat offspring. Castration reduces and stabilizes endogenous androgens and androgen-derived estrogens. The same protocol was applied to female offspring (Durrer et al. 2005; Maerkel et al. 2005, 2007) to compare target gene responses in both sexes. At 10 weeks, male offspring were taken out of several litters of control and 4-MBC–exposed groups (0.01, 0.1, and 0.33 g/kg chow) and castrated under anesthesia 150 µL/100 g body weight sc of an acqueous solution containing 12.5% Hypnorm (Janssen-Cilag, Baar, Switzerland), 12.5% Domitor (Pfizer, Zurich, Switzerland) and 2.5% atropin (Pharmacy of the University Hospital, Zurich, Switzerland). After 2 weeks recovery (age 12 weeks), they received an sc injection of E_2 [10 or 50 µg/kg in dimethylsulfoxide (DMSO)] or vehicle (DMSO) (injection volume 1 µL/g body weight) and were decapitated 6 hr later. Effects on prostate were studied in ventral lobe because dissection was faster and could be better fitted to a strict time schedule. Animals from the same litters were studied under baseline conditions.

Quantification of mRNA by real-time reverse transcription–polymerase chain reaction (RT-PCR). Real-time RT-PCR was performed as described previously (Durrer et al. 2005). Prostate tissues were homogenized in lysis tissue buffer from RNeasy-mini kit (QIAGEN, Valencia, CA, USA) by polytron rotor-stator homogenizer; total RNA isolated according to manufacturers's specifications (QIAGEN). Genomic DNA was digested by DNase-I (QIAGEN). Quality and concentration of RNA were determined by measuring absorbance at 260 and 280 nm, and RNA integrity was confirmed by an ethidium bromide-stained 2.5% agarose gel. RNA was stored at –80°C. For reverse transcription, 10 µg RNA was used in a total volume of 100 µL containing 1 × TaqMan RT buffer (Applied Biosystems, Rotkreuz, Switzerland), 5.5 mM $MgCl_2$, 500 µM of each deoxynucleoside triphosphate, 2.5 µM random hexamer primers, 0.4 µM RNase inhibitor, and 1.25 µL MultiScribe reverse transcriptase (Applied Biosystems). The mixture was incubated 10 min at 25°C followed by 30 min RT at 48°C and 5 min RT inactivation at 95°C. RT samples were frozen at –80°C. Primers and probes were designed using Primer Express 2.0 software (Applied Biosystems) and were synthesized by Microsynth (Balgach, Switzerland). Sequences are summarized in Table 2. RT-PCR cycle

Table 1. Treatment schedules.

Experimental group	Postnatal F_1	Adult F_1
1. Baseline study		
1.1. Untreated control A 4-MBC 7, 24, 47 mg/kg/day	Developmental landmarks, puberty, no experimental manipulations	Age 12 weeks: Organ weights; mRNA and protein analyses in ventral and dorsolateral prostate
1.2. Untreated control B 4-MBC 0.7 mg/kg/day		
2. Estrogen challenge[a]		
2.1. Untreated control A 4-MBC 7, 24 mg/kg/day	Postnatal day 70: Castration	Postnatal day 84: Subcutaneous injection of estradiol (10 or 50 µg/kg)[b] or vehicle; Sacrifice after 6 hr; mRNA in ventral prostate
2.2. Untreated control B 4-MBC 0.7 mg/kg/day		

[a]Offspring for estrogen challenge experiments (n = 8/group) were taken out of litters from the baseline study. [b]Untreated control B and 4-MBC 0.7 mg/kg/day injected only with 10 µg/kg estradiol or vehicle.

Table 2. Forward primers (FP), reverse primers (RP), and TaqMan probes with corresponding GenBank accession number.

Gene (GenBank accession no.)[a]	Primer and probe sequences (5′-3′)
Androgen receptor (M20133)	
FP	5′-CGGAAGGGAAACAGAAGTATCTATG-3′
RP	5′-GGAGACGACACGATGGACAA-3′
Probe	5′-CCAGCAGAAATGATTGCACCATTGATAAATTTC-3′
Estrogen receptor-α (NM_012689)	
FP	5′-CCAAAGCCTCGGGAATGG-3′
RP	5′-AGCTGCGGGCGATTGAG-3′
Probe	5′-TCGTTCCCTTGGATCTGGTGCAACAA-3′
Estrogen receptor-β (NM_012754)	
FP	5′-TTGGTGTGAAGCAAGATCACTAGAG-3′
RP	5′-AACAGGGCTGGCACAACTG-3′
Probe	5′-CCACTAAGCTTCCTCTTCAGTGTCTCTCTGTTTACA-3′
Insulin-like growth factor-1 (M15481)	
FP	5′-GGCCCAGCGCCACA-3′
RP	5′-TGTTTCCTGCACTTCCTCTACTTG-3′
Probe	5′-TGACATGCCCAAGACTCAGAAGGAAGTACA-3′
Cyclophilin (M19533)	
FP	5′-TGTGCCAGGGTGGTGACTT-3′
RP	5′-0TCAAATTTCTCTCCGTAGATGGACTT-3′
Probe	5′-CCACCAGTGCCATTATGGCGTGT-3′

[a]Gene names and accession numbers are from GenBank (http://www.ncbi.nlm.nih.gov/).

parameters were an initial denaturating step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min in ABI PRISM 7700 Sequence Detector (Applied Biosystems).

Sequence Detector Software SDS 2.0 (Applied Biosystems) was used for data analysis. mRNAs were quantitated according to the standard curve method and normalized to cyclophilin. Cyclophilin was chosen as reference because it was found not to be affected by manipulations of gonadal hormones (Durrer et al. 2005). mRNA levels of ventral prostates of intact and castrated rats were compared with the comparative C_t method. ΔC_t is the difference of the C_t values of the target gene and of the reference gene (cyclophilin). The difference between paired tissue samples (control and treated) is then obtained as $\Delta\Delta C_t = [\Delta C_t$ (control tissue) $- \Delta C_t$ (treated tissue)]. For a given gene, the *n*-fold differential expression of a test sample compared with the control was expressed as $2^{-\Delta\Delta Ct}$ (Applied Biosystems 2001).

Western blot analysis. Protein levels were analyzed in the same tissue homogenates as used for mRNA analysis. Because RNA stability is most critical, tissue was homogenized in RNA lysis buffer. Protein concentration was determined by Bradford method (Bio-Rad Laboratories, München, Germany) using lyophilized bovine serum albumin (Fluka, Buchs, Switzerland) as standard. For acetone precipitation of protein from buffer RLT lysates, the QIAGEN protocol (http://www1.qiagen.com/literature/protocols/pdf/RY22.pdf) was used. After addition of 200 mM dithiothreitol (Fluka), samples (20 µg protein) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Precise Protein Gels; PIERCE Biotechnology, Inc., Rockford, IL, USA). The running buffer contained 100 mM Tris base, 100 mM Hepes, 3 mM SDS. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in a Trans-Blot apparatus (Bio-Rad Laboratories). After transfer, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.5% (v/v) Tween 20 (blocking buffer). Membranes were then incubated overnight with the first antibody diluted in blocking buffer. Anti-ER-α and anti-androgen receptor (AR) (ABR-Affinity BioReagents, Golden, CO, USA) were diluted 1:5000, ER-β, steroid receptor coactivator-1 (SRC-1) and anti-nuclear receptor co-repressor (N-CoR) (Santa Cruz Biotech, Santa Cruz, CA, USA) 1:2,000, 1:2,500, and 1:1,000 respectively, anti-actin (Chemicon Int., Temecula, CA, USA) 1:100,000, anti-cyclophilin A (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) 1:10,000. After three washes with blocking buffer diluted 1:5 with TBS, membranes were incubated for 2 hr with the appropriate secondary antibodies conjugated to horseradish peroxidase. Proteins were detected using chemiluminescence (Pierce). Densitometrical analyses were performed using AIDA-2D software (Raytest, Pittsburgh, PA, USA). Molecular masses were verified by using prestained SDS-PAGE standards [Broad Range (myosin 204 Da to aprotinin 7 Da)] from Bio-Rad Laboratories. Binding specificity of antibodies for the respective proteins was examined with a negative control sample. Results are expressed as ratio of the protein of interest to actin to correct for possible differences of protein loading between samples. Values of treated samples were expressed as percentage of the mean of the control group loaded on the same blot (control A and 7- or 24-mg/kg group, control B and 0.7-mg/kg group). Gels run in duplicate for the same two treatment groups yielded analogous effect patterns. All proteins showed specific signals (Figure 1)with molecular weights of the expected size described in the literature and according to the manufacturer's instructions.

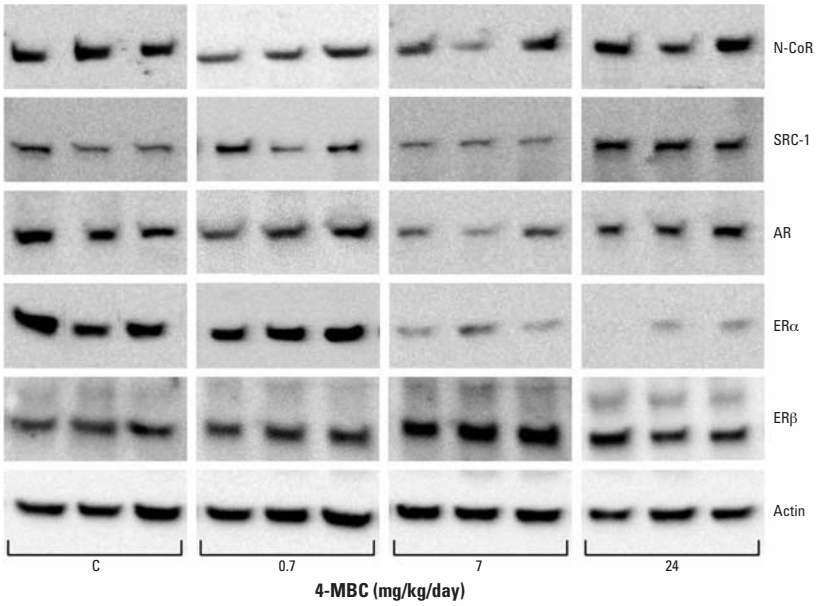

Figure 1. Representative Western blots from dorsolateral prostate of untreated and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed 12-week-old rat offspring. Actin = reference protein. Molecular masses of proteins verified by prestained SDS-PAGE standards (Broad Range, Bio-Rad Laboratories).

Table 3. Analysis of actin as reference protein in dorsolateral prostate (Western blots)

	Actin protein density (% of control) [a]	Density ratio actin protein/cyclophilin protein [a]
Control	100.0 ± 15.2 (7)	
4-MBC 0.7 mg/kg/day	112.6 ± 19.4 (7)	
Control	100.0 ± 27.1 (7)	0.79 ± 0.24 (7)
4-MBC 7 mg/kg/day	90.7 ± 17.2 (7)	0.77 ± 0.14 (7)
Control	100.0 ± 10.1 (7)	0.64 ± 0.16 (7)
4-MBC 24 mg/kg/day	97.9 ± 27.9 (7)	0.62 ± 0.12 (7)

[a]Mean ± SD, number of dorsolateral prostate samples. Control and one treatment group were analyzed in one Western blot. No significant group differences in one-way ANOVA.

Several possible reference proteins were studied, including β-tubulin used on uterus (Durrer et al. 2005). Actin was found to be more appropriate for prostate tissues. The reliability of actin was checked by three approaches: *a*) General specificity checks; with the antibody used, a clear and consistent protein signal was obtained (Figure 1). *b*) Absolute values of actin did not differ between controls (CON) and 4-MBC-exposed groups analyzed in the same blot of dorsolateral prostate (Table 3). *c*) Actin was compared with cyclophilin, which is not influenced by estrogen (Durrer et al. 2005), in the same blot of dorsolateral prostate with samples from control and treated animals. The ratio cyclophilin protein/actin protein was the same in controls and 4-MBC-exposed groups (Table 3). Thus, actin was equivalent to cyclophilin in the present setup. The latter could not be used as reference because its molecular weight differs considerably from that of target proteins.

Statistics. Series A (untreated control A, 7, 24, 47 mg/kg 4-MBC) and B (untreated control B, 0.7 mg/kg 4-MBC) were analyzed separately because they had been conducted during different periods. Individual mRNA

was normalized to cyclophilin mRNA and expressed as percentage of the respective control (A, B). Body weight, organ weights, and mRNA were analyzed by one-way ANOVA with Bonferroni pair-wise comparisons and Dunnett's multiple comparison test for series A (control A vs. treated groups), and by Student's *t*-test for series B (control B vs. 0.7 mg/kg group). Western-blot gels comprised a lane of controls (A or B) and a lane of samples of one treatment group. Values of treated samples were expressed as percentage of the mean of control samples of individual gels. Differences between control (A or B) and treated group of individual gels were assessed by Student's *t*-test. Preputial separation and vaginal opening exhibited positively skewed distributions also in controls, and were analyzed with non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. (GraphPad Prism 4; GraphPad Software Inc., San Diego, CA, USA).

Results

Onset of puberty and reproductive organ weights. 4-MBC was administered to rats in chow, to the parent generation before mating, during pregnancy and lactation, and to the offspring until adulthood (12 weeks) (Table 1). The treatment delayed puberty onset in males (preputial separation) at and above 7 mg/kg (Table 4, not analyzed with 0.7 mg/kg). Two separate experiments started in summer 2001 and spring 2002 yielded identical results (Table 4). Body weight at onset of puberty was at control level in males, but slightly reduced in females. Adult body weights of 4-MBC–exposed offspring were in the control range (Table 5; Durrer et al. 2005).

Ventral prostate weight decreased in adult offspring exposed to 7–47 mg/kg 4-MBC (Table 5). Investigations in 13-month-old offspring showed that 4-MBC reduced both, ventral as well as dorsal + lateral prostate weight (Appendix). Absolute testis weight was slightly increased (Table 5). Epididymis and seminal vesicle weights were unaffected except for relative epididymis weight at 47 mg/kg. Higher doses of 4-MBC (24, 47 mg/kg) increased thyroid weight (Maerkel et al. 2007). Liver weight remained unchanged (data not shown).

Effect of pre- and postnatal exposure to 4-MBC on gene expression in prostate lobes of adult rat offspring. mRNA and protein levels were determined in the same samples of ventral prostate and dorsolateral prostate (dorsal + lateral lobes) in 12-week-old offspring under baseline conditions (Table 6; Figures 1 and 2). AR mRNA and protein

Table 4. Onset of puberty in male (preputial separation) and female rat offspring (vaginal opening)

		4-MBC		
	Control[a]	7 mg/kg/day	24 mg/kg/day	47 mg/kg/day
Preputial separation (PND)				
Individual animals	45.71 ± 0.82 (52)	46.41 ± 0.84 (41)*	47.55 ± 1.70 (44)#	48.97 ± 2.35 (29)#
Litters	45.95 ± 0.52 (17)	46.35 ± 0.65 (12)	47.82 ± 1.51 (13)#	49.32 ± 1.88 (12)#
Body weight at preputial separation (g)	180.3 ± 10.0 (52)	183.0 ± 11.5 (41)	179.4 ± 10.2 (44)	181.0 ± 11.3 (29)
Vaginal opening (PND)				
Individual animals	39.36 ± 2.43 (53)	38.65 ± 2.03 (51)	38.02 ± 1.89 (33)	39.44 ± 1.85 (25)
Body weight at vaginal opening (g)	123.0 ± 10.8 (53)	117.2 ± 9.65 (51)*	112.9 ± 8.60 (33)**	112.2 ± 7.70 (25)**

Combined data are from two series (summer 2001, spring 2002). Means of individual rats for preputial separation series 1/series 2: control 45.76/45.63; 4-MBC: 7 mg/kg/day, 46.89/46.28; 24 mg/kg/day, 47.72/47.42; 47 mg/kg/day, 48.79/49.13.
[a]Data are presented as mean ± SD (number of animals or litters). Different from control: *$p < 0.05$, **$p < 0.01$, #$p < 0.001$.

Table 5. Reproductive organ weights and body weight in 12-week-old male rat offspring after pre- and postnatal exposure to 4-MBC.

Treatment[a]	Mean individual body weight[b] (g)	Mean individual organ weights[b]		Mean body weight of litters[b] (g)	Mean organ weights of litters[b]	
		Absolute weight (g)	Relative weight[c]		Absolute weight (g)	Relative weight[c]
		Ventral prostate	Ventral prostate		Ventral prostate	Ventral prostate
Untreated control A	351.3 ± 36.7 (34)	0.326 ± 0.068 (34)	0.466 ± 0.093 (34)	354.0 ± 30.8 (13)	0.330 ± 0.060 (13)	0.466 ± 0.077 (13)
4-MBC (7 mg/kg/day)	370.2 ± 23.6 (21)	0.284 ± 0.064 (21)*	0.385 ± 0.088 (21)**	371.4 ± 21.6 (11)	0.296 ± 0.049 (11)	0.398 ± 0.060 (11)*
4-MBC (24 mg/kg/day)	368.8 ± 27.4 (24)	0.283 ± 0.050 (24)*	0.385 ± 0.063 (24)**	371.5 ± 21.6 (11)	0.287 ± 0.042 (11)	0.391 ± 0.053 (11)*
4-MBC (47 mg/kg/day)	352.4 ± 45.0 (23)	0.246 ± 0.057 (23)**	0.349 ± 0.068 (23)#	350.9 ± 39.5 (9)	0.249 ± 0.040 (9)**	0.356 ± 0.045 (9)**
Untreated control B	399.3 ± 21.8 (18)	0.371 ± 0.051 (18)	0.465 ± 0.062 (18)	397.9 ± 14.0 (7)	0.367 ± 0.029 (7)	0.461 ± 0.037 (7)
4-MBC (0.7 mg/kg/day)	399.7 ± 20.3 (15)	0.381 ± 0.062 (15)	0.476 ± 0.065 (15)	400.4 ± 10.0 (6)	0.384 ± 0.041 (6)	0.478 ± 0.044 (6)
		Testis[d]	Testis		Testis[d]	Testis
Untreated control A		1.462 ± 0.099 (32)	1.683 ± 0.153 (32)		1.453 ± 0.068 (12)	1.656 ± 0.127 (12)
4-MBC (7 mg/kg/day)		1.538 ± 0.064 (21)*	1.719 ± 0.127 (21)		1.551 ± 0.073 (11)	1.675 ± 0.091 (11)
4-MBC (24 mg/kg/day)		1.587 ± 0.117 (24)#	1.725 ± 0.103 (24)		1.597 ± 0.105 (11)**	1.722 ± 0.090 (11)
4-MBC (47 mg/kg/day)		1.594 ± 0.140 (23)#	1.821 ± 0.120 (23)#		1.580 ± 0.137 (9)*	1.813 ± 0.077 (9)**
Untreated control B		1.513 ± 0.050 (18)	1.519 ± 0.091 (18)		1.513 ± 0.032 (7)	1.525 ± 0.058 (7)
4-MBC (0.7 mg/kg/day)		1.481 ± 0.108 (14)	1.481 ± 0.105 (14)		1.498 ± 0.076 (6)	1.486 ± 0.066 (6)
		Epididymis[d]	Epididymis		Epididymis[d]	Epididymis
Untreated control A		0.440 ± 0.073 (27)	0.637 ± 0.078 (27)		0.444 ± 0.073 (10)	0.635 ± 0.060 (10)
4-MBC (7 mg/kg/day)		0.456 ± 0.097 (20)	0.639 ± 0.121 (20)		0.476 ± 0.088 (9)	0.654 ± 0.122 (9)
4-MBC (24 mg/kg/day)		0.458 ± 0.069 (24)	0.622 ± 0.085 (24)		0.462 ± 0.067 (11)	0.621 ± 0.078 (11)
4-MBC (47 mg/kg/day)		0.396 ± 0.081 (21)	0.566 ± 0.084 (21)*		0.391 ± 0.076 (8)	0.564 ± 0.074 (8)
Untreated control B		0.511 ± 0.055 (11)	0.599 ± 0.107 (11)		0.508 ± 0.052 (4)	0.627 ± 0.039 (4)
4-MBC (0.7 mg/kg/day)		0.495 ± 0.027 (9)	0.617 ± 0.032 (9)		0.500 ± 0.028 (5)	0.618 ± 0.024 (5)
		Seminal vesicle[d]	Seminal vesicle		Seminal vesicle[d]	Seminal vesicle
Untreated control A		0.377 ± 0.080 (21)	0.529 ± 0.102 (21)		0.387 ± 0.045 (10)	0.540 ± 0.072 (10)
4-MBC (7 mg/kg/day)		0.407 ± 0.055 (18)	0.550 ± 0.058 (18)		0.406 ± 0.058 (11)	0.550 ± 0.059 (11)
4-MBC (24 mg/kg/day)		0.385 ± 0.065 (23)	0.524 ± 0.085 (23)		0.389 ± 0.052 (10)	0.521 ± 0.068 (10)
4-MBC (47 mg/kg/day)		0.348 ± 0.048 (17)	0.481 ± 0.041 (17)		0.343 ± 0.041 (7)	0.482 ± 0.029 (7)
Untreated control B		0.437 ± 0.039 (9)	0.552 ± 0.047 (9)		0.432 ± 0.042 (5)	0.545 ± 0.038 (5)
4-MBC (0.7 mg/kg/day)		0.437 ± 0.037 (12)	0.552 ± 0.060 (12)		0.444 ± 0.027 (6)	0.562 ± 0.041 (6)

[a]Series A: control A and 7, 24, 47 mg/kg/day 4-MBC. Series B: control B and 0.7 mg/kg/day 4-MBC. [b]Data presented are mean ± SD (number of animals or number of litters). [c]Relative weights: ventral prostate = absolute (g) /body weight (g) × 500. Testis = absolute (left + right)/body weight × 200. Epididymis and seminal vesicle = absolute (left + right)/body weight × 250. [d]Testis, epididymis, seminal vesicle weights = (left + right)/2. Different from control A: *$p < 0.05$, ** $p < 0.01$, #$p < 0.001$.

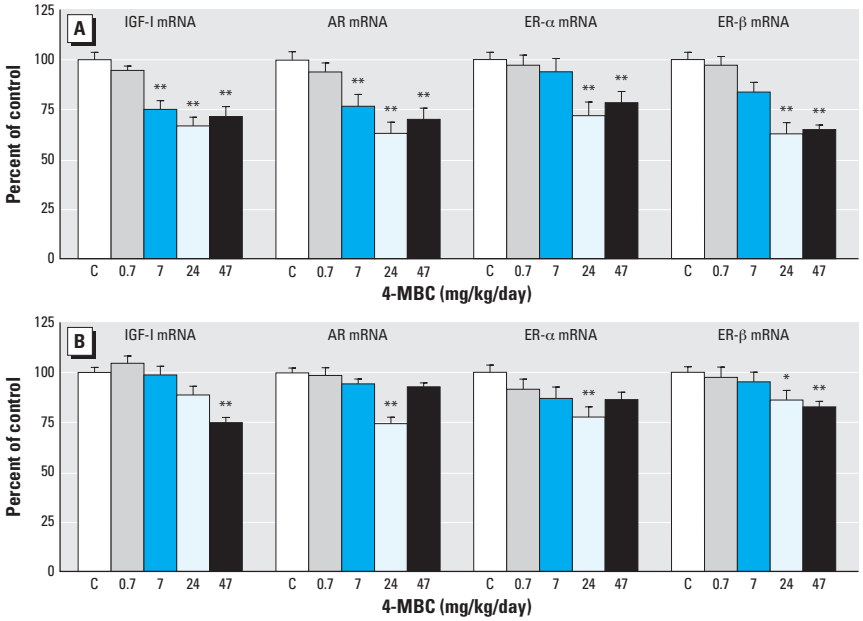

Figure 2. Levels of mRNAs encoding for insulin-like growth factor-1 (IGF-1), androgen receptor (AR, ER-α , and ER-β in dorsolateral (*A*) and ventral (*B*) prostate of untreated adult (12-week-old) rat offspring and offspring exposed to 4-MBC; 0.7, 7, 24, 47 mg/kg/day in chow. Real-time RT-PCR values were normalized to cyclophilin and are expressed as percentage of the mean of the corresponding untreated control (C) [mean ± SE, $n = 8$, pooled controls A ($n = 8$) + B ($n = 8$)].
Different from corresponding control A (for 7, 24, 47 mg/kg) or B (for 0.7 mg/kg), $^{*}p < 0.05$, $^{**}p < 0.01$.

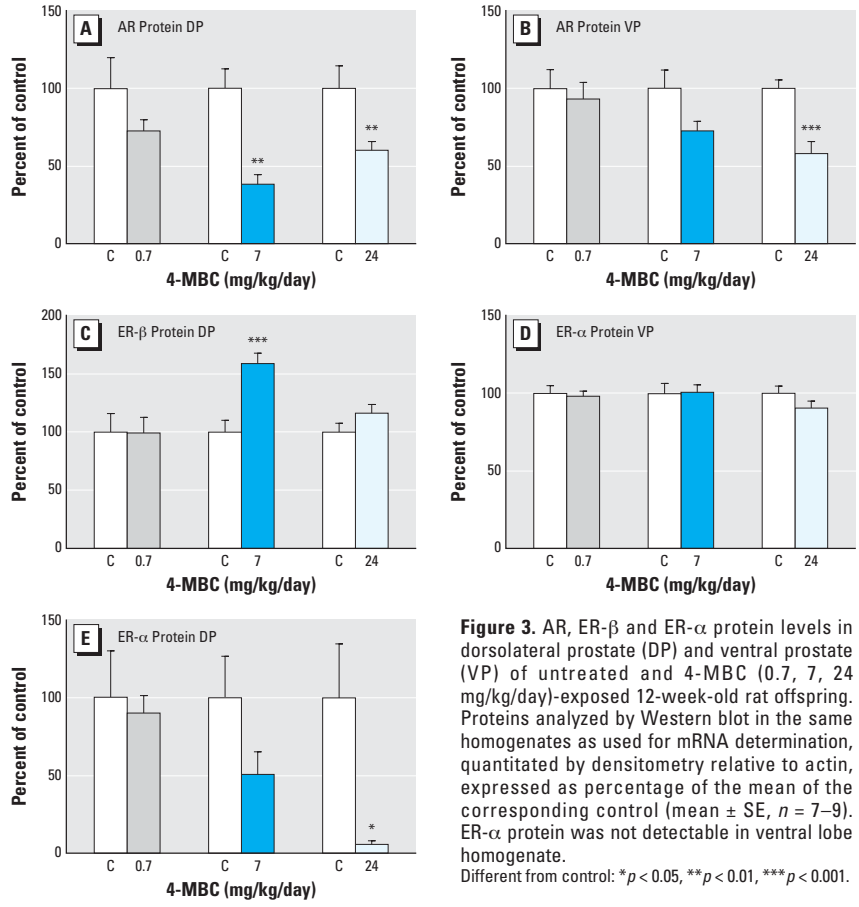

Figure 3. AR, ER-β and ER-α protein levels in dorsolateral prostate (DP) and ventral prostate (VP) of untreated and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed 12-week-old rat offspring. Proteins analyzed by Western blot in the same homogenates as used for mRNA determination, quantitated by densitometry relative to actin, expressed as percentage of the mean of the corresponding control (mean ± SE, $n = 7$–9). ER-α protein was not detectable in ventral lobe homogenate.
Different from control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

levels were reduced (Figures 2, 3). ER-α expression was lower in ventral than dorsolateral prostate (Table 6; Lau et al. 1998). 4-MBC down-regulated ER-α mRNA in both tissues. A decrease in ER-α protein was observed in dorsolateral prostate; in ventral prostate, the protein was not detectable. ER-β mRNA was down-regulated in both prostate parts, but protein was increased in dorsolateral prostate at 7 mg/kg and unchanged in ventral prostate (Figure 3). The increase in ER-β protein coincided with the lowest level of AR protein. Insulin-like growth factor 1 (IGF-1) mRNA was dose dependently down-regulated. Effects of 4-MBC on all four genes were greater in dorsolateral prostate.

Acute response to E_2 in adult castrated rat offspring. To examine changes in responsiveness of estrogen-regulated genes to E_2 in ventral prostate, offspring from different litters of untreated control groups A and B and 0.7, 7, and 24 mg/kg/day 4-MBC treatment groups were castrated at 70 days of age, and after 2 weeks recovery, injected with E_2 (10 or 50 μg/kg, sc) 6 hr before sacrifice (Table 1).

Effects of castration were analyzed by comparing mRNA levels in ventral prostate of intact adult control offspring with levels in vehicle-injected castrated control offspring (comparative C_t method). Castration increased ER-α, AR, and IGF-1 mRNAs and reduced ER-β mRNA (Table 7). Transcript levels of castrated, vehicle-injected groups, which represent the reference level of E_2 challenge experiments, did not differ significantly between controls and 4-MBC–exposed groups, except for ER-β mRNA at 0.7 mg/kg (Table 8).

Effect of injection of E_2. IGF-1 mRNA was down-regulated in ventral prostate of castrated controls 6 hr after injection of E_2 (10 or 50 μg/kg, sc), relative to the vehicle group (Figure 4A). An analogous effect of E_2 was observed in 4-MBC–exposed males, except for one injection group (10 μg/kg E_2, 24 mg/kg 4-MBC). However, the magnitude of the suppressive effect of E_2 on IGF-1 mRNA was dose dependently reduced compared with the E_2 effect in controls (Figure 4B). AR mRNA was down-regulated by both doses of E_2 in controls, whereas 4-MBC-exposed animals responded to only 10 μg/kg E_2 (Figure 5A). The suppression of AR mRNA by 50 μg/kg E_2 was significantly smaller in 4-MBC–exposed offspring.

ER-α mRNA was down-regulated by E_2 (Figure 5B). ER-β mRNA levels were not significantly changed by E_2 in controls, whereas a small repressive effect was visible in some 4-MBC–exposed groups (signficant at 24 mg/kg after 10 μg/kg E_2. Figure 5C). In contrast to AR and IGF-1, effects of E_2 on mRNAs encoding for ER-α and ER-β did not differ significantly between control and 4-MBC–exposed offspring.

Co-regulator protein levels. In 4-MBC–exposed female littermates, SRC-1 protein levels were decreased in uterus (Durrer et al. 2005). This was correlated with reduced inductive effects of E_2 on progesterone receptor and IGF-1. SRC-1 protein levels were not significantly affected in prostate (Figure 6), but N-CoR protein levels were reduced after exposure to 0.7, 7 and 24 mg/kg 4-MBC (Figure 6), to 55.3, 64.5, and 76.1% in dorsolateral prostate, and 102.4, 85.8, and 75.8% in ventral prostate, respectively.

Discussion

The present study indicates that exposure to the UV filter 4-MBC throughout ontogeny until adulthood affects male sexual development in rats, puberty, reproductive organ weights, expression, and estrogen sensitivity of estrogen-regulated genes in prostate and N-CoR protein levels. Gene expression is also altered in uterus of female littermates (Durrer et al. 2005) and in brain of the same animals (Maerkel et al. 2005, 2007).

Developmental exposure to estrogenic substances can delay puberty in male rats (Biegel et al. 1998; Masutomi et al. 2003), but data are conflicting [for genistein, see Masutomi et al. (2003) and Wisniewski et al. (2003)], and in part negative (Putz et al. 2001b; Takagi et al. 2004). Observations on hepatic testosterone hydroxylase in prepubertal males indicate that effects of neonatal estrogen may vary with dose (Putz et al. 2001b). The delay of male puberty by 4-MBC, which binds to ER (Schlumpf et al. 2004a) but not AR (Ma et al. 2003), may be linked with its estrogenic activity, but it should be noted that the chemical also interacts with the thyroid axis (Maerkel et al. 2007; Schlumpf et al. 2004b; Schmutzler et al. 2004). The changes in testis weight decrease at PN14 (Schlumpf et al. 2001b), and the slight increase that occurs in adulthood may also be linked with estrogenic activity, as testis weight was reduced on day 18 after neonatal diethylstilbestrol (Atanassova et al. 2000) and increased in adulthood after low-dose neonatal E_2 (Putz et al. 2001b).

Most conspicuous changes were noted in prostate with a marked decrease in weight as demonstrated for ventral prostate and in an additional study also for dorsolateral prostate. Prostate development appears to be enhanced by slightly supernormal estrogen levels (Nagel et al. 1997; Timms et al. 1999; vom Saal et al. 1997) but inhibited by higher estrogen levels (Prins 1992; Putz et al. 2001a). Activational responses to androgens are similarly enhanced or reduced (Naslund and Coffey 1986; Rajfer and Coffey 1978). Because 4-MBC does not bind to AR (Ma et al. 2003), the reduction in prostate weight probably resulted from its estrogenic activity. The 4-MBC-induced reduction of AR may also be involved. Effects of 4-MBC resembled developmental low-dose actions of estrogen with respect to testis but high-dose actions with respect to puberty and prostate weight, suggesting differences in sensitivity of target systems.

The changes in gene expression in prostate of adult offspring may have resulted from effects of 4-MBC during ontogeny, from ongoing exposure in adulthood or from a combination of both. mRNAs encoding for IGF-1, AR, ER-α, and ER-β, and AR and ER-α proteins were down-regulated in dorsolateral and ventral prostate, with statistical significance of changes in mRNA and protein at the same dose levels. The reduction of ER-β mRNA is reminiscent of observations after neonatal estrogen treatment (Prins et al. 1998). Dose–response curves appear to be monotonic, with the exception of ER-β protein and possibly N-CoR in dorsolateral prostate. The increase in ER-β protein at 7 mg/kg, with mRNA at control level, is not explainable. It might reflect combined effects on receptor synthesis and degradation. 4-MBC exhibits partial agonist features (Schlumpf et al. 2001a), and partial agonists can inhibit proteasomal ER degradation (Fan et al. 2004; Laios et al. 2003), but this should

Table 6. mRNA levels in prostate lobes of adult control rat offspring.

mRNA[a]	Ventral lobe (VP) (%)[b]	Dorsolateral lobe (DP) (%)[b]
IGF-1	100 ± 13.57	394.15 ± 68.69*
ER-α	100 ± 24.87	5,512.75 ± 1137.25*
ER-β	100 ± 15.68	114.12 ± 18.20
AR	100 ± 6.10	70.68 ± 10.49*

[a]mRNA levels calculated by the comparative C_t method. [b]Percentage of intact VP control (mean ± SD , $n = 8$). *Different from control, $p < 0.05$.

Table 7. mRNA levels in ventral prostate of intact and castrated adult male control offspring.

mRNA[a]	Intact control (%)[b]	Castrated control (%)[b]
IGF-1	100 ± 13.57	315.19 ± 46.17*
ER-α	100 ± 24.87	2,014.31 ± 520.33*
ER-β	100 ± 15.68	34.51 ± 7.79*
AR	100 ± 6.11	330.62 ± 72.09*

[a]mRNA levels calculated by the comparative C_t method. [b]Percentage of intact control (mean ± SD , $n = 8$). *Different from control, $p < 0.05$.

Table 8. mRNA levels in adult control and 4-MBC-exposed male rat offspring after castration and vehicle injection.

mRNA	Untreated control A	4-MBC (7 mg/kg/day)[a]	4-MBC (24 mg/kg/day)[a]	Untreated control B	4-MBC (0.7 mg/kg/day)[a]
IGF-1	100 ± 14.65	85.38 ± 17.81	77.70 ± 13.03	100 ± 9.89	98.56 ± 21.14
ER-α	100 ± 25.83	65.58 ± 16.55	124.82 ± 24.78	100 ± 24.46	97.14 ± 25.87
ER-β	100 ± 22.57	87.25 ± 17.84	82.21 ± 12.10	100 ± 22.64	148.66 ± 28.26*
AR	100 ± 21.80	102.86 ± 16.05	116.19 ± 24.88	100 ± 19.36	106.26 ± 15.18

[a]mRNA as percentage of control A or control B. Mean ± SD, $n = 8$. *Different from control, $p < 0.05$.

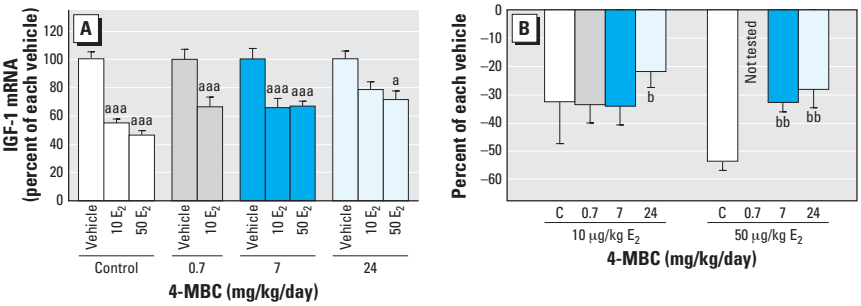

Figure 4. (*A*) Insulin-like growth factor-1 (IGF-1) mRNA 6 hr after sc injection of E_2 [10 μg/kg (10 E_2) or 50 μg/kg (50 E_2)] or vehicle in ventral prostate of castrated 12-week-old rat offspring from control and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed groups. Values normalized to cyclophilin, as percentage of the corresponding vehicle-injected group (mean ± SE, $n = 8$). (*B*) Magnitude of down-regulation of IGF-1 mRNA by E_2 in ventral prostate of castrated adult rat offspring of control and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed groups. Values normalized to cyclophilin, as percentage of the corresponding vehicle-injected group [mean ± SE, $n = 8$, control = pooled control groups A ($n = 8$) + B ($n = 8$)].

Separate statistics for 0.7-mg/kg group vs. control B, and 7- and 24-mg/kg groups vs. control A. Different from corresponding vehicle-injected group: $^{a}p < 0.05$, $^{aaa}p < 0.001$. Magnitude of E_2 effect different from control: $^{b}< 0.05$, $^{bb}p < 0.01$.

influence also ER-β in ventral prostate and ER-α levels.

Lowest AR protein levels coincided with the ER-β peak in dorsolateral prostate. Mice lacking ER-β exhibit AR up-regulation in prostate (Cheng G et al. 2002). Because 4-MBC is a preferential ER-β ligand (Schlumpf et al. 2004a), actions mediated by ER-β may have contributed to reduced AR expression. AR mRNA is also down-regulated by genistein, considered to be an ER-β ligand (Fritz et al. 2002). However, expression of AR probably was affected by additional mechanisms such as impairment of prostate development through effects on ER-α. Neonatal exposure to estrogenic substances can lead to reduced or enhanced AR expression in adulthood (Prins and Birch 1995; vom Saal et al. 1997). The difference has been ascribed exclusively to dosage, but preference of a ligand for ER-α or ER-β might also influence the effect pattern. Provided they were already present in early life, the down-regulation of AR, ER-α and IGF-1 may all have contributed to impaired prostate development. ER-α has been associated with proliferation and branching (Asano et al. 2003; Omoto et al. 2005), IGF-1 also influences prostate development and appears to be involved in enhanced branching in response to estrogen (Gupta 2000; Torring et al. 1997).

Endocrine regulation may be disturbed by changes in estrogen sensitivity of target genes. To obtain information on this aspect, the effect of E_2 on gene expression in ventral prostate was studied in an acute challenge experiment in littermates of the males studied under baseline conditions. Endogenous hormone levels were reduced and stabilized by castration. Castration-induced changes in AR, ER-α and ER-β and IGF-1 mRNA expression corresponded to previous observations (Asano et al. 2003; Bacher et al. 1993; Nickerson et al. 1999). Acute E_2 injection elicited the expected down-regulation of AR mRNA, which may be mediated via ER-β (Weihua et al. 2002). A suppressive effect of E_2 on IGF-1 has previously been observed in prostate in the absence of testosterone (Nellemann et al. 2005) and is also indicated by observations with the antiestrogen ICI 182,780 (Huynh et al. 2001). Exposure to 4-MBC reduced the suppressive effect of E_2 on both, AR and IGF-1 mRNA expression, indicating a reduction of sensitivity of the two genes to the natural estrogen. In contrast, down-regulation of ER-α and ER-β by E_2 remained unaffected. The direction of change in estrogen sensitivity induced by 4-MBC was the same in uterus (Durrer et al. 2005).

Since ER-β mRNA was not significantly changed in vehicle-injected castrated animals by 7 or 24 mg/kg 4-MBC, the reduced repressive effect of E_2 on AR cannot be ascribed to changes ER-β (Weihua et al. 2002). Sensitivity to steroid hormones also depends on availability of steroid receptor coregulators. 4-MBC–induced changes of coactivator SRC-1 levels in uterus (Durrer et al. 2005) prompted us to investigate nuclear receptor coregulator expression in prostate. In addition to the coactivator SRC-1 (McKenna and O'Malley 2002), the corepressor N-CoR was studied because effects of hydroxytamoxifen (Lavinsky et al. 1998; Shang et al. 2000) suggested the possibility of effects of 4-MBC, which shows partial ER agonist characteristics (Schlumpf et al. 2001a). The 4-MBC–induced down-regulation of N-CoR protein might explain reduced gene repression by E_2, which recruits N-CoR (Stossi et al. 2006), but N-CoR levels in castrated rats could not be determined because the small amount of ventral prostate homogenate allowed only mRNA analyses. Reduced baseline AR levels in presence of reduced sensitivity to E_2 and N-CoR levels indicate that the chronic condition probably resulted from a combination of multiple

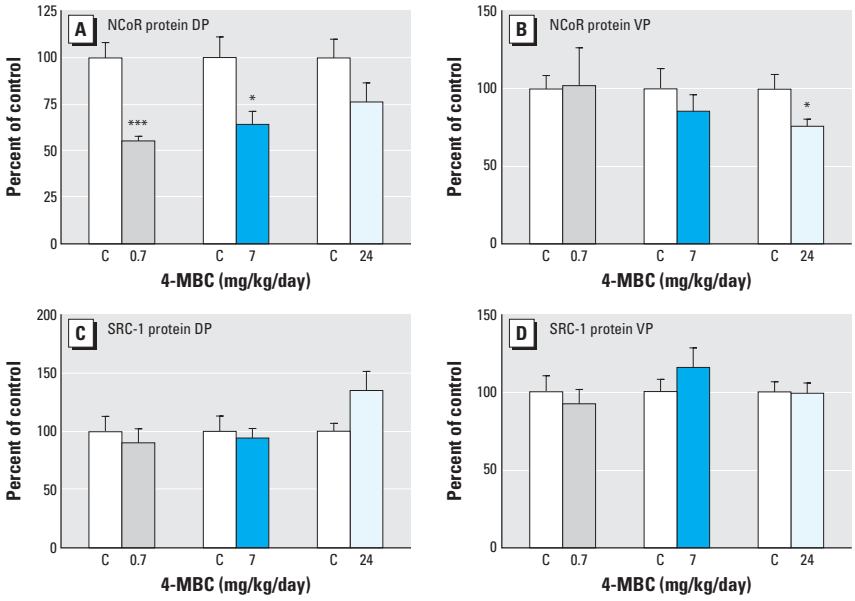

Figure 6. Steroid receptor coactivator-1 (SRC-1) and nuclear receptor corepressor (N-CoR) protein levels (Western-blot) in dorsolateral (dorsal + lateral) prostate (DP) and ventral prostate (VP) of untreated and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed 12-week-old rat offspring. Proteins analyzed in the same homogenates as used for mRNA determination, quantitated by densitometry relative to actin, expressed as percentage of the mean of the corresponding control (mean ± SE, n = 7–9).
Different from control: *$p < 0.05$, ***$p < 0.001$.

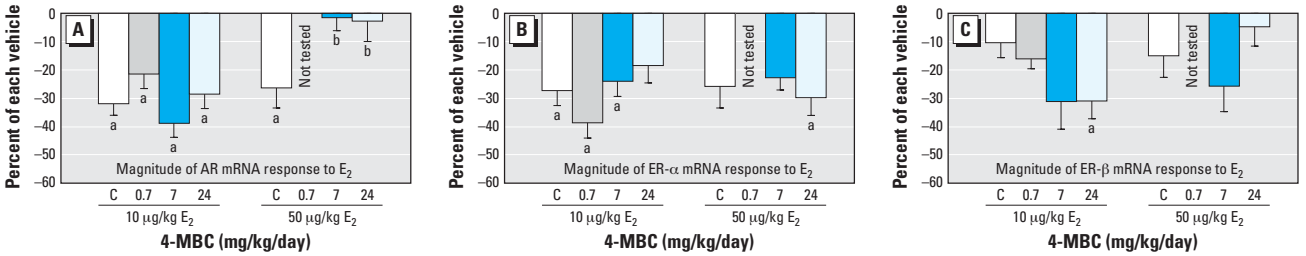

Figure 5. Magnitude of acute suppression of mRNAs encoding for AR (*A*), ER-α (*B*) and ER-β (*C*) in ventral prostate of castrated adult rat offspring from control (C) and 4-MBC (0.7, 7, 24 mg/kg/day)-exposed groups 6 hr after sc injection of E_2 [10 μg/kg (10 E_2) or 50 μg/kg (50 E_2)]. Values normalized to cyclophilin, as percentage of the corresponding vehicle-injected group [mean ± SE, n = 8; control = pooled control groups A (n = 8) + B (n = 8)].
Separate statistical analysis for 0.7-mg/kg group vs. control B, and 7- and 24-mg/kg groups vs. control A: [a]Significant suppression of mRNA by E_2 compared with vehicle, $p < 0.05$. [b]Magnitude of suppression by E_2 different from untreated control, $p < 0.05$.

developmental and adult processes (Weihua et al. 2001; Woodham et al. 2003).

Our data demonstrate that N-CoR is a target of endocrine disruptors. N-CoR protein is reduced by acute administration of E_2 in MCF-7 cells (Frasor et al. 2005) and by chronic hydroxytamoxifen in a mouse tumor model (Lavinsky et al. 1998). The effect of E_2 was attributed to up-regulation of ubiquitin ligase and targeting of N-CoR for proteasomal degradation. Down-regulation of N-CoR may have implications for gene expression on a broad scale because N-CoR is recruited by many transcription factors, it also interacts with AR (Cheng S et al. 2002; Hodgson et al. 2005). Changes in N-CoR levels may be relevant for prostate ontogeny and adult function.

In conclusion, our data indicate that pre- and postnatal exposure of rats to 4-MBC interferes with male sexual development. Classical end points showed a lowest observed adverse effect level (LOAEL) of 7 mg/kg and a NOAEL of 0.7 mg/kg; molecular end points (N-CoR) a LOAEL of 0.7 mg/kg. These doses are 30 and 3 times above an estimated human exposure level of 0.23 mg/kg (Scientific Committee on Cosmetic Products and Non-Food Products 1998). Thus, the margin of safety (MOS = NOAEL/exposure × 100) may not be reached. However, we think that risk considerations should be based on a comparison of internal exposure levels in humans and experimental animals. Adipose tissue levels of 4-MBC in adult rat offspring were 449 ng/g lipid at 7 mg/kg (Schlumpf et al. 2004b), close to fish levels (44–166 ng/g lipid, Balmer et al. 2005). To obtain information on internal human exposure, a monitoring study on human milk is presently being conducted.

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